(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 299 108 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication of patent specification: **18.05.94**

(51) Int. Cl.5: **C12N 15/00**, C12N 15/53, C12N 15/81, C12N 1/14, C12N 9/26, C12P 21/02

(21) Application number: **87110417.0**

(22) Date of filing: **17.07.87**

(54) DNA-molecules coding for FMDH control regions and structured gene for a protein having FMDH-activity and their uses.

(43) Date of publication of application:
18.01.89 Bulletin 89/03

(45) Publication of the grant of the patent:
18.05.94 Bulletin 94/20

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(56) References cited:
EP-A- 0 183 071

NUCLEIC ACIDS RESEARCH, vol. 13, no. 9, May 1985, pages 3063-3082, Oxford, GB; A.M. LEDEBOER et al.: "Molecular cloning and characterization of a gene coding for methanol oxidase in Hansenula polymorpha"

NUCLEIC ACIDS RESEARCH, vol. 13, no. 9, May 1985, pages 3043-3062, Oxford, GB;Z.A. JANOWICZ et al.: "Cloning and characterization of the DAS gene encoding the major methanol assimilatory enzyme from the methylotrophic yeast Hansenula polymorpha"

(73) Proprietor: **Rhein Biotech Gesellschaft für biotechnologische Prozesse und Produkte mbH**
Erkrather Strasse 230
D-40233 Düsseldorf(DE)

(72) Inventor: **Hollenberg, Cornelius P.**
Chopinstrasse 7
D-4000 Düsseldorf(DE)
Inventor: **Janowicz, Zbigniew**
Adalbert Stifterstrasse 49
D-4006 Erkrath 1(DE)

(74) Representative: **Patentanwälte Grünecker, Kinkeldey, Stockmair & Partner**
Maximilianstrasse 58
D-80538 München (DE)

EP 0 299 108 B1

AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 47, no. 11, November 1983, pages 2547-2554, Tokyo, JP; J.J. ALLAIS et al.: "Oxidation of methanol by the yeast Pichia pastoris. Purification and properties of the formate dehydrogenase"

BIOTECHNOLOGY, vol. 5, no. 5, mai 1987, pages 479-485, New York, US; J.M. CREGG et al.: "High-level expression and efficient assembly of hepatitis B surfaceantigen in the methylotrophic yeast, Pichia pastoris"

PROCEEDINGS OF THE 4TH EUROPEAN CONGRESS ON BIOTECHNOLOGY, Amsterdam, 14th-19th June 1987, vol. 2: Abstracts/Extended abstracts, page 4, Elsevier Science Publishers B.V., Amsterdam, NL; A.A. HINNEN: "Yeast as an expression system for heterologous proteins"

NUCLEIC ACIDS RESEARCH, vol. 15, no. 9, 11th May 1987, pages 3859-3876, IRL Press Ltd, Oxford, GB; J.F. TSCHOPP et al.: "Expression of the lacZ gene from two methanol-regulated promoters in Pichia pastoris"

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 80, Nov. 1983, pages 6872-6876, Washington, D.C., US; M. ROA et al.: "Biosynthesis of peroxisomal enzymes in the methylotrophic yeast Hansenula polymorpha"

MOLECULAR AND CELLULAR BIOLOGY, vol. 5, no. 5, May 1985, pages 1111-1121, American Society for Microbiology, Washington, D.C., US; S.B. ELLIS et al.:"Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast Pichia pastoris"

JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 261, no. 28, October 1986, pages 12942-12947, The American Society of Biological Chemistry, Baltimore, M.D. US; A.P. SHUBER et al.: "Cloning, expression, and nucleotide sequence of the formatede hydrogenase genes from Methanobacterium formicicum"

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 83, July 1986, pages 4650-4654, Washington, D.C. US; F. ZINONI et al.: "Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli"

"Gene und Klone" by E.L. Winnacker (1984), Verlag chemie Ed. p.404

Description

The present invention is directed to a DNA fragment comprising a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having format dehydrogenase (FMDH) activity, recombinant vectors containing said fragment, microorganisms containing said vectors and a process for producing a substance using the microorganisms according to the present invention.

During the last decade, several yeast strains were isolated which are able to utilize methanol as an only carbon and energy source. Until recently the studies were limited to the enzymatic level and concerned mainly two species, namely Hansenula polymorpha and Candida boidinii.

The enzymatic studies revealed that in methylotrophic yeasts methanol is oxidised via formaldehyde and formate to CO_2 by methanol oxidase (MOX), formaldehyde dehydrogenase (FMD) and formate dehydrogenase (FMDH), respectively. H_2O_2 which is generated during the first oxidation stop is degraded by catalase. C1 compound is assimilated by transketolase reaction of xylulose-5-(P) and formaldehyde, the latter being derived from the dissimilatory pathway. The reaction is catalysed by dihydroxyacetone synthase (DHAS).

Growth of methylotrophic yeast on methanol is accompanied by changes in total protein composition. There are 3 major and about 5 minor proteins newly synthesized. Further, the growth on methanol is accompanied by appearance of huge peroxisomes. These organelles bear some of the key enzymes involved in methanol metabolism, namely, MOX, DHAS and catalase (1). The other two methanol enzymes FMD and FMDH, are cytoplasmic proteins. In methanol grown cells, the enzymes FMDH, MOX, and DHAS constitute up to 40% of total cell protein. The methanol utilisation pathway is highly compartmentalised and the integration of these reactions is very complex.

The methanol dissimilatory enzymes are regulated by glucose catabolite repression/derepression mechanism (2). Methanol has an additional inductive effect increasing the expression level by the factor of 2-3. In H. polymorpha, assimilatory DHAS enzyme follows this general regulation scheme, however, during growth on limiting amounts of glucose, derepression, an additional post transcriptional mechanism, plays a role in the regulation.

Recently, 3 genes encoding peroxisomal enzymes were cloned from H. polymorpha and Pichia pastoris and the analysis of nucleotide sequences of MOX genes from H. polymorpha (3) and P. pastoris (4) and DAS gene, which encodes DHAS from H. polymorpha (5) revealed that a cleavable signal sequence is not required for the transport of MOX and DHAS into the peroxisome.

The promoters of some methanol genes are very efficient and their way of regulation is favourable to the industrial application. The expression of foreign proteins can be enhanced and placed under stringent control. The large amounts of proteins (MOX, DHAS) thus produced by methylotrophic yeast are stored in the peroxisomes. The understanding of this mechanism will help to solve some problems of the stability of foreign proteins in yeast.

In the field of industrial biotechnology, there is a need for microbiological regulation systems by which large amounts of a particularly desired protein can be produced under stringent control. Although there are already promoter/terminator systems available which can be used in genetic engineering systems for controlling the amount of proteins to be produced, there is still a strong need for further regulatory systems to be available since it has turned out that, in biological systems, it is advantageous to provide more systems so that the most effective one can be chosen. The present systems are far from being efficient, especially when stringent regulation and high mitotic stability is required.

It was, therefore, an object of the present invention to provide a more effective and a very easily controllable regulatory system.

The advantage of the present invention is given by providing a DNA fragment, which comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment.

To start more comprehensive studies on basic research and biotechnological aspects of methanol utilisation, the gene encoding the cytoplasmic methanol key enzyme FMDH was cloned. The sequence of this 1020 bp long gene and its regulatory regions have been cloned. FMDH is regulated at transcriptional level by glucose catabolite repression/deprepression/methanol induction mechanism.

The DNA-fragment according to this invention is extremely useful in the biotechnology industry because of the above discussed characteristic that the expression of foreign proteins can be enhanced and placed under Stringent control.

The DNA fragments according to the present invention may be modified by recombinant DNA technology techniques as known in the art, resulting in a modified promoter, which still retains its promoter function.

A preferred embodiment of the DNA-molecule of this invention is shown in Fig. 5.

Examples for the use of the FMDH regulatory sequences of the present invention are combinations of said DNA sequences with foreign genes encoding hepatitis B virus S1-S2-S amtigen and hepatitis B virus S antigen α-amylase from S. casteilii and glucoamylase from S. casteilii or invertase from Saccharomyces cerevisiae.

The DNA- fragments of this invention may further be combined to DNA-sequences which are coding for secretory signals, such as Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins, methanol oxidase and dihydroxyacetone synthase, Schwanniomces castelli α-amylase and glucoamylase signals, or Saccharomyces cerevisiae α-factor and invertase signals.

Preparation of the DNA-fragments coding for control regions and the structural gene for protein having FMDH activity may be obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

Recombinant vectors can be prepared which contain the DNA fragments according to this invention either as such, coding for the regulatory regions and/or structural genes for FMDH protein and may be combined to further DNA sequences as discussed above. Recombinant vectors for the purposes of transferring DNA sequences into an expression system are commonly used in the art and may be properly chosen. For example, the λ Charon 4A phage may carry the described DNA- fragments.

As micro-organisms which are suitable for the expression of the desired genes also may be selected from known micro-organisms in the art which are adapted for recombinant DNA technologies. Micro-organisms, however, who are able to tolerate high concentrations of foreign proteins are preferred.

Most preferred are micro-organisms of the genera Candida, Hansenula or Pichia.

The mentioned micro-organisms are able to produce the desired substances either by integration of the DNA-molecules of this invention into the chromosom of the micro-organism or by maintaining the DNA-molecules on an extra chromosomal DNA-molecule via episomal vectors.

The proteins coded by foreign genes combined to the DNA-fragments of the present invention and being produced by the transformed micro-organisms can be obtained by culturing said micro-organisms in a manner known in the art and recovering the proteins as is also standard knowledge in the art.

The invention is now presented, in a more detailed manner, by the following specification and figures. The figures show:

Figure 1: Analysis of protein crude extracts and in vitro translation products by SDS-polyacrylamid gel electrophoresis.
Lanes 7-9:Coomassie Blue stained gel; protein crude extracts from induced, derepressed and uninduced cells, respectively. Lane 10, purified FMDH. Lanes 1-3:^{35}S-labelled in vitro translation products of mRNA isolated from induced, uninduced cells and fractionated mRNA enriched in FMDH mRNA species, respectively. Lane 4; immunoprecipitation of translation products from lane 1. Lane 5; translation of hybrid-selected mRNA. Lane 6: immunoprecipitation of translation products from lane 5.

Figure 2: Restriction map of DNA fragment encompassing the FMDH gene.
The arrow shows the direction of transcription.

Figure 3: S1-mapping;
Lanes M1, 1, 2, 3, 4, M2, a, b, c, d, separation on alkaline agarose gel. Lanes 5, M3-separation on 6% polyacrylamide gel/8M urea. Lanes M1-M3-MW markers. Lanes 1, 2-total protection (1) of 4.1 kb Eco-RI/Hind III fragment (2) encompassing the gene. Lanes 3, 4-protection of 3'-end labelled 1.4 kb Bam HI/Hind III fragment; 3-protected band; 4-1.4 kb intact band. Lane 5-protection of 1 kb Bam HI/Pst I fragment with a single label at Bam HI site. Lanes a, b, c, d-protection of 3'-end labelled DNA fragment containing part of the gene by mRNA preparation isolated from: induced, derepressed (1% glycerol), stationary phase of 3% glucose and mid-log phase of 3% glucose cultures, respectively.

Figure 4: Sequencing strategy - schematic representation.
DNA fragments containing the gene were subjected to Bal31 digestion and the resulting fragments subcloned into M13 and/or pUC type vectors. The fragments were sequenced by Sanger and in the case of doubts Maxam-Gilbert

methods.

Figure 5a: Muclectide sequence of FMDH gene and its 5', 3' control regions.

Figure 5b: Nucleotide sequence of FMDH gene and its 5', 3' control regions.

Figure 5c: Nucleotide sequence of FMDH gene and its 5', 3' control regions.

Figure 6: Plasmid containing the fusion of bacterial β-lactamase gene with FMDH promoter.

Figure 7: Plasmid containing the hepatitis S-gene; HARS - H. polymorpha autonomous replicating sequence; URA3 - S. cerevisiae gene; FMDH-promoter (-9 type promoter).

Figure 8: Western blot-stained by peroxidase/protein A method. Polyclonal antibodies (not clarified) were used in this experiment:

Lane a : LR9 growth on methanol

Lane i : transformant w/o S-gene

Lanes k, l, m : transformants with S-gene grown on glucose (repression)

Lanes b, c, d, e, f, g : different transformants with S-gene grown on methanol

Lanes n, o : 500 and 450 ng purified HSBAg, respectively

Figure 9: Plasmid expressing α-amylase gene; symbols are the same as in Fig. 7.

Figure 10: Growth of transformants on medium containing methanol (induction). Enzyme activity (U/ml) was measured in medium and in cells (intracellular enzyme level). The latter value was expressed as corresponding to 1 ml of medium.

Figure 11: The formation of halo after applying on the plate 50 ul of the medium from transformants (upper row) and from control untransformed strain LR9 (lower row).

Strains, media, vectors:

Thermophilic, homothallic strain of H. polymorpha (ATCC 34438) was used. Yeast was grown at 37 • C on minimal YNB medium as described (3, 5). Induction of methanol utilisation system was achieved by growth in minimal medium containing 1% methanol; growth on 3% glucose minimal medium resulted in repression of the system.

E. coli L90; C600recA, hsdM, araB, was used for transformation;
E. coli JM103, thi, strA, supE, endA, sbcB, hsdR, F'traD36, proAB, lacI, ZM15, and
E. coli KH802 gal, met, supE, were used as host for phage M13 and for λ-vector Charon 4A, respectively. Plasmid DNA and RF M 13 were isolated by scaled-up alkaline minilysates methods (6) followed by CsCl ultracentrifugation.

λ-vector Charon 4A and Charon 4 recombinant clones were isolated by scaled-up plate lysate methods (6).

H. polymorpha total DNA of the size greater than 50 kb was isolated from spheroplasts as previously described (5).

Charon 4 H. polymorpha DNA library was constructed by ligating partially EcoRI digested H. polymorpha DNA with Charon 4 arms as described previously (5).

PolyA mRNA from H. polymorpha and analysis of the mRNA by an in vitro cell free rabbit reticulocyte system is described previously (5).

mRNA labelling: mRNA was partially fragmented by mild alkaline treatment (7) and labelled at the 5'-end with γ-^{32}P-ATP (Amersham).

The differential plague filter hybridisation was performed essentially as described in (12). Recombinant phages were plated to about 3,000 pfu per plate. Plaques from each plate were blotted into a set of 5-6 replica nitro-cellulose filters (BA85, Schleicher and Schüll). The filters were hybridized to appropriate ^{32}P-mRNA or ^{32}P-DNA probes in 5 x SSPE. 50%, formamide, containing additionally 150 ug/ml tRNA, 10 ug/ml poly A, 5 x Denhardt's solution, 5 ug/ml rRNA from H. polymorpha isolated as described in (5, 6).

S1 mapping experiments were performed essentially as described by Favarolo et at. (8). S1 nuclease from NEN at concentration 1,000 units/ml was used.

Hybrid selection technique was performed as described by Büneman et at. (9). Briefly, DNA from recombinant subclones was covatently bound to DPTE derivative of Sephacryl S-500. Total mRNA was then hybridized with DNA/S-500 matrix. mRNA species not complementary to the immobilized DNA were washed out under very stringent conditions (5, 9). Hybridized mRNA was eluted with H_2O at 100 • C. Hybrid selected mRNA was then translated in cell-free system, and the translation products analyzed by immunoprecipitation as described previously.

Sequence analysis: Different overlapping fragments derived from the exonuclease Bal31 digestion of DNA fragments encompassing FMDH gene were cloned into M13 phages mp9, mp8 and into plasmid pUC12, puC13. The subcloned fragments were sequenced by Sanger et al. (10) and Maxam-Gilbert (11) methods.

Formate dehydrogenase was purified to homogeneity from methanol grown H. polymorpha cells as described elsewhere. Antibodies against FMDH, denaturated form, were raised in rabbits according to standard procedures.

Identification of mRNA species encoding FMDH In vitro translation products of total mRNA isolated from cells grown on 3% glucose (repression) or 1% methanol (induction) were analyzed on SDS-PAGE gels. Figure 1 shows the comparison of in vitro translation products of mRNA from induced (lane 1), not induced (lane 2) cells, as well as the immunoprecipitates of the first preparation with specific antibodies directed against FMDH (lane 4). In addition, the electrophoretic patterns of crude protein extracts from 1% methanol, 0.5% glycerol/0.1% glucose (derepression) and 3% glucose cultures were compared with the electrophoretic mobility of purified FMDH (lanes 7, 8, 9 and 10 respectively).

The results obtained clearly identified the FMDH protein positionon SDS-PAGE, and indicate that FMDH protein and its mRNA are predominant species in cells grown on methanol (induction). The position of two other predominant proteins, MOX and DHAS, is also indicated. Fig. 1 also points out that considerable expression is achieved under derepressed conditions (lane 8) and that 3% glucose represses the enzymes of methanol utilisation system. Above conclusions enabled us to isolate through sucrose gradient centrifugation mRNA fraction enriched in mRNA encoding FMDH (lane 3) in order to use it for screening procedure.

Screening for FMDH gene

The H. polymorpha DNA bank in Charon 4 phage was screened by differential plaque hybridisation (Materials and Methods) with radioactive ^{32}P-labelled mRNA from induced, not induced cells and with ^{32}P-mRNA from a fraction enriched in FMDH mRNA (Fig. 1, lane 3). Additionally, replica filters were hybridized with ^{32}P-DNA probes from clones encoding MOX and DAS genes (3, 5). The latter was done to identify and eliminate the clones encoding the two other strongly inducible genes. Desired phages were selected and their DNA further characterized.

Characterisation of recombinant clones

The initial identification of a clone was achieved by hybrid selection technique, restriction mapping and establishing the size of the mRNA encoded by a given clone.

Hybrid selection

DNA from Charon4 recombinant clone JM was covalently bound to DPTE S-500 matrix, mRNA complementary to JM clone was selected and its in vitro translation products analyzed. Fig. 1 shows that the hybrid selected mRNA gives upon in vitro translation a major peptide product of the same elecrophoretic mobility as FMDH peptide (lane 5). When peptides from lane 5 were precipitated with specific antibodies (lane 6), a major band of a size of FMDH and additional weak band are visible. In control experiment with not-induced mRNA not detectable mRNA of FMDH character was selected by this technique. The presence of additional weak bands visible in lane 5 and 6 are probably artefacts of the used hybrid selection technique.

These data strongly suggest that clone JM contains FMDH gene.

Restriction map and the size and direction of transcription

Restriction map of clone JM and its subclones is shown in Fig. 2. DNA fragments encompassing the gene were identified by hybridizing the Southern blots with ^{32}P-labelled induced mRNA.

8.5 kb EcoRI H. polymorpha DNA fragment from clone JM contains a gene. A further analysis allowed to subclone the gene and its presumptive regulatory regions on HindIII/EcoRI 4.1 kb fragments in pBR325.

S1 mapping

Non-radioactive HindIII/EcoRI 4.1 kb fragment from plasmid p3M1 was isolated and annealed with induced and not-induced mRNA. The size of DNA protected by its cognate mRNA against the action of nuclease S1 was analyzed by agarose electrophoresis followed by Southern blotting and hybridization with appropriate ^{32}P-DNA in order to visualize the fragment. Fig. 3, lane 1 shows that induced mRNA protects 1.2 kb long DNA fragment. This indicates that the gene codes for a protein of about 35-37,000 daltons. This value was found for the FMDH protein. Since in this MW region FMDH is the only strongly inducible protein, this result supports the identification of the gene.

3' end of the gene, transcription direction and the amount of FMDH transcript

Two fragments containing the gene, 1.0 kb BamHI/PstI and 1.4 kg HindIII/BamHI, were isolated and a 3' end label was introduced at BamHI site. Only the label on the right (Fig. 3, lane 3-4), 1.4 kb HindIII/BamHI fragment was protected by annealing with mRNA indicating the direction of transcription from left to right (arrow in Fig. 2). This size of the band (lane 4) indicates that the 3' end of the gene is located 850 bp to the right of the BamHI site. This type of experiment was also used to roughly establish the amount of FMDH

mRNA species in total polyA+ mRNA isolated from cells grown under different conditions. A known amount of ^{32}P-3' end labelled DNA containing part of the gene was hybridized with varying amounts of mRNA. At DNA excess conditions, the radioactivity present in a band protected against S1 by a given amount of mRNA is a measure of the quantity of FMDH mRNA in the preparation. The data indicate that FMDH mRNA contributes about 7% ± 1% and 3% to 4% of total polyA+ mRNA in preparation from induced and derepressed growth condition respectively. Fig. 3, lanes a, b, c, d, shows the comparison of intensity of the DNA band resulting from S1 experiments where 3 ug of DNA was hybridized with 10 ug of total polyA+ mRNA. It is also clearly visible that in mid-log phase of 3% glucose (repression) cultures, only negligible amounts of FMDH transcript is visible whereas the same culture at stationary phase shows already considerable amounts of transcript. this is a good example of derepression phenomenon - in stationary phase, glucose is exhausted.

5' end of the gene

1.0kb BamHI/PstI fragment with a single 5' end label at BamHI yielded upon S1 mapping the multiple bands ranging from 255-265 bp (Fig. 3, lane 5). The comparison of this value with sequence data indicated that transcription starts around position -12 from the first ATG. The lain band shows the start at "A" surrounded by pyrimidine track.

Nucleotide sequence

The nucteotide sequence of FMDH gene and encompassing region was determined by Sanger (10) and Maxam-Gilbert methods (11). The fragments to be sequenced were generated by deleting with Bal31 DNA containing the gene. Fig. 4 shows that all regions of the gene were sequenced several times in both directions. In case of doubts, M13 method data were corrected by data obtained by Maxam-Gilbert methods. The nucleotide sequence is presented in Fig. 5. The gene contains an open reading frame (ORF) of 1,020 nucleotides and code for a protein of 340 Da. The protein MW, calculated from these data, is 35,700 Da which agrees well with the values obtained by SDS-PAGE of purified protein. The gene was conclusively identified as FMDH by comparing the N-end of the gene as derived from DNA sequence with the data obtained by NH-end analysis of the purified protein.

5'-3' end regions

In the 5'-control regulatory region of eukaryotes, a consensus sequence -3A(9)XX1AUG4GX6py was reported to be required in efficiently transcribed and translated genes (12, 13). In FMDH gene, the rule is only partly followed where the sequence -3AUG + 1AUG + 4AX + 6A is present. The first ATG is proceeded by stop codons in all reading frames. The sequence CTATAAATA involved in eukaryotes in the initiation of transcription is found at position -40. Other features assumed to play a role in transcriptional control in yeast S. cerevisiae like CAACAA or CACACA (12) not present in FMDH.

In most of the yeasts studied until now, the gene 3' end region contains characteristic sequences which, according to some authors, play a role in proper termination of transcription and serve as polyadenylation signals (14, 15). Zared and Sherman (16), and Bennetzen and Hall (17) assumed that a sequence T-rich...TAG...TAGT(or TATGT)...AT...TTT or T...TAAATAA...A(or G)...T...A..AT play these roles. In FMDH gene, similarity to these consensus Sequences is rarely found. When looking for some potential signals, some repeating sequences were found. Sequences TTGGA and TAGG repeat twice. AAATATAA, similar to animal polyadenylation signal, is located 30 bp downstream from the end of ORF.

Example 1:

In order to be able to study the functional regions of FMDH 5' upstream region, a series of deletion of this region was isolated. First, to obtain the promoter without the structural gene, a pUC type plasmid containing the 1.4 kb Bam HI fragment was subjected to Bat31 exonuclease treatment after the plasmid was linearised at a proper point. At the beginning, attention was focused on the promoter fragment which had the deletion at the position -5 from the first ATG; the fragment is called "-5 promoter". Also "-9" deleted promoter was used in some experiments.

The "-5 promoter" was fused to the open reading frame of the bacterial β-lactamase gene (Bla). The gene was used in the laboratory as a very suitable model for studying the expression of foreign protein under the control of yeast promoters.

The signal sequence of the β-lactamase was not present in the construction obtained, thus enabling the measurement of enzyme activity in yeast protein extracts. The fused DNA fragment was cloned into the plasmid containing H. polymorpha autonomously replicating sequence (HARS1) (Fig. 6), and S. cerevisiae Ura3 gene which serves as a marker for H. polymorpha transformation. The amount of β-lactamase produced in H. polymorpha transformants was measured by the enzymatic and immuno-tests. Table 1 shows the synthesis of β-lactamase under the control of FMDH promoter in cells grown in different media

(different carbon sources).

Table 1 shows that the isolated FMDH promoter is properly and stringently controlled by repression/derepression/induction mechanism. The estimation of the amount of synthesized protein shows that the system of this invention is characterised by very efficient transcription and translation of the foreign protein. In the control experiment, β-lactamase was expressed in S. cerevisiae under the control of a strong S. cerevisiae PDC (pyruvate decarboxylase) promoter on 2-µm plasmid (50 copies per cell). The values obtained were lower than in the case of H. polymorpha by a factor of 5-6.

TABLE 1

Production of β-lactamase						
clone	enzymatic test (U/mg protein)			immuno-test (% of total cell protein)		
	GLU	GLYC	Met-OH	GLU	GLYC	Met-OH
Ir 45	30	4,000	15,000	-	3-4	6-8
L 5	70	10,000	28,000	-	6-8	10-12
GLU - growth on 3% glucose (repression) GLIC - growth on 1% glicerol (derepression) Met-OH - growth on 1% methanol (induction)						

In all cases, cells from late logarithmic phase were taken for measurement. The plasmid containing the fusion has 50-60 copies per cell.

Example 2:

Expression of genes encoding Hepatitis B surface antigens (HSBAg) under the control of FMDH promoter

1. Construction of the plasmid expressing the hepatitis proteins.

Hepatitis B 1,2 kb DNA fragment encodes a long S2-S1-S-protein (pre-s), which after processing (removal of S2-S1-part) is converted into the S-protein. Viral envelope consists of both proteins.

For our expression experiments we have used the 1,2 kb fragment as well as a shorter part of this DNA which encodes only S-protein. The latter is also able to form antigenic pseudo viral particles.

We have inserted both hepatitis S-gene into our universal vector. As shown in Fig. 1 and scheme 1 the vector contains autonomous replication sequence (HARS), URA3 gene from S. cerevisiae as a selective marker and H. polymorpha promoter followed by short linker. After the S-gene we have placed DNA fragment derived from H. polymorpha MOX gene exhibiting the transcription terminator function. Fig. 7 shows the construction containing the S-gene.

2. Transformation of H. polymorpha and screening for clones expressing HSBAg.

H. polymorpha URA3 mutant LR9 was transformed with the above described plasmids. The yeast transformants were then immediately screen for the expression of HBSAg using polyclonal antibodies. As an immuno-screening we have used Western blotting (peroxidase-protein A or to improve sensitivity ^{125}J - protein A). The screening procedure was considerably impeded by the strong cross-reactivity of the sera with H. polymorpha crude extract proteins. We were, however, able to show the expressed antigem.

Fig. 8 shows the Western blotting Protein extracts from cells transformed with hepatitis gene grown on methanol and shows an additional antigenic band having the expected MW of S-protein. The control extracts from transformants grown on glucose (repression of FMDH promoter) do not have this band. The results shown in Fig. 8 are coming from transformants containing FMDH -9 promoter i.e. promoter derived by deleting the DNA fragment encompassing the promoter function till position -9 from the first ATG.

We analysed also by testing S1-nuclease mapping mRNA produced in our transformants. The results indicate that transformants are producing a lot of S-gene mRNA species and that the transcription is stringently controlled by repression/derepression/induction mechanism.

The above results were confirmed by positive RIA TEST of protein extracts derived from transformed cells. In the test the momoclonal antibodies directed against native S-protein were used.

Example 3:

Expression and secretion of α-amylase from Schwanniomyces castelli in H. polymorpha under the control of FMDH promoter.

To study the possibility of expressing in H. polymorpha a secretory protein we have chosen α-amylase gene from yeast S. castellii. The gene encodes the 56 kd protein which in S. castellii is totally secreted into the medium; this secretory process is accompanied by glycosilation of the protein.

We have inserted EcoRI fragment encompassing the structural gene and its terminator into our expression plasmid (Fig. 9).

H. polymorpha was transformed with this plasmid and the transformants were tested for the expression and secretion of α-amylase using a starch degradation test (halo formation on starch-iodine plates) or enzyme kinetik test kit (α-amylase Merkotest A).

The results clearly show that α-amylase is produced under control of FMDH promoter. Moreover, the protein is secreted into the medium. Fig. 10 shows that in mid-log phase about 90% of the protein is secreted into the medium. Starch-iodine plate test confirmed these results (Fig. 11).

The data also show that it is possible to get a high expression level under derepressed conditions. This feature of the system is especially very valuable and important for biotechmological applications, i.e. the synthesis of foreign proteins can begin without addition of methanol as inducer simply by exhausting glucose in the medium and/or by the addition of glycerol. A system that can be handled in such an easy way by simultaneously providing a very effective expression yielding amounts of proteins applicable in the biotechnological industry has not been provided earlier.

In separate studies it has been shown that the other H. polymorpha promoters like MOX and DAS do not respond so strongly to derepression signals. In the case of DAS promoter, the expression under derepressed condition is additionally decreased by post-transcriptional control.

REFERENCES

1. Douma, A.L., Veenhuis, M., Koning, W., Evers, M., and Harder, W.,
Arch of Microbiol., (1985), 145, 237.
2. Roggenkamp, R., Janowicz, z., Stanikowski, B. and Hollemberg, C.P.
(1984) Mol. Gem. Genet. 194, 489-493.
3. Ledeboer, A.M., Maat, J., Visser, C., Bos, J.W. Verrips, C.T., Janowicz, Z., Eckart, M., Roggenkamp, R. and Hollenberg, C.P.
(1985) Nucleic Acid Res., 3063.
4. Ellis, S.B., et al. Isolation of alcohol oxidase and two other methanol regulatory genes from the yeast Pichia pastoris. Molecular and Cellular Biology (1985) 5:1111-21.
5. Janowicz, Z.A., Eckart, M.R., Drewke, C., Roggenkamp, R.O., Hollemberg, C.P., Maat, J., Ledeboer, A.M., Visser, C. and Verrips, C.T.,
Nucl. Acid. Res. (1985) 13, 3043.
6. Williams, B.G. and Blattner, F.R. (1979) J. Virol. 29, 555.
7. Goldbach, R.W., Borst, P., Bollen-de-Boer, J.E. and van Bruggen, E.F.J.
(1978) Biochem. et Biophys. Acta 521, 169-186.
8. Favarolo, J., Treisman, R. and Kamen, R.
(1980) Methods in Enzymology 65, 718.
9. Bünemann, H., Westhoff, P. and Herrmann, R.G.
(1982) Nucl. Acid. Res. 10, 7163-7178.
10. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.j.H. and Roe, B.A.
(1980) J. Mol. Biol. 143, 161-178.
11. Maxam, A.M. and Gilbert,
Methods Enzymol. (1980) 65, 499.
12. van Dijken, S.P., Veenhuis, M. and Harder, W.
(1982) Ann. N.Y. Acad. Sci. 386, 200-216.
13. Kozak, M.
(1981) Nucl. Acid Res. 9, 5233-5252.

14. Sahm, H.
(1977) Adv. Biochem. Eng. 6,77-103.
15. Veenhuis, M., van Dijken, J.P. and Harder, W.
(1983) Adv. Microbiol. Physiol. 23,2-76.
16. Zaret, K.S. and Sherman, F.
(1982) Cell 28, 563-573.
17. Bennetzen, J.H. and Hall, B.D.
(1982) J. Biol. Chem. 257, 3018-3025.

Claims

Claims for the following Contracting States : BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. A DNA fragment characterized in that it comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment.

2. The DNA fragment according to claim 1, characterized in that the gene codes for a wild type FMDH protein.

3. The DNA fragment according to any one of claims 1 or 2, characterized in that it has been modified by recombinant DNA technologies, while retaining its promoter function.

4. The DNA fragment according to claim 1, characterized in that it comprises the 5' region of the nucleotide sequence shown in Figure 5.

5. The DNA fragment according to any one of claims 1 to 4, characterized in that it further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.

6. The DNA fragment according to claim 5, characterized in that the said foreign gene is selected from genes encoding:
   (a) Hepatitis B Virus S1-S2-S antigen
   (b) Hepatitis B Virus S-antigen
   (c) alpha-amylase from Schwanniomyces castellii
   (d) glucoamylase from Schwanniomyces castellii
   (e) invertase from Saccharomyces cerevisiae.

7. The DNA fragment according to any one of claims 1 to 6, characterized in that it comprises a DNA sequence coding for a secretory signal.

8. The DNA fragment according to claim 7, characterized in that the secretory signal is selected from: Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α-amylase and glucoamylase signals, Saccharomyces cerevisiae α-factor and invertase signals.

9. The DNA fragment according to any one of claims 1 to 8, characterized in that it has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

10. A recombinant vector, characterized in that it contains the DNA fragment according to any one of claims 1 to 9.

11. A microorganism characterized in that it comprises a vector according to claim 10.

12. The microorganism according to claim 11, characterized in that it is a methylotrophic yeast.

13. The microorganism according to claim 12, characterized in that the yeast is selected from the genera Candida, Hansenula or Pichia.

14. The microorganism according to any one of claims 11 to 13, characterized in that it has received the vector according to claim 10 by transformation.

15. The microorganism according to any one of claims 11 to 14, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.

16. The microorganism according to any one of claims 11 to 15, characterized in that it tolerates high concentrations of foreign protein.

17. A process for producing a useful substance, characterized in that a microorganism according to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a manner known in the art.

Claims for the following Contracting State : AT

1. A DNA fragment characterized in that it comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment.

2. The DNA fragment according to claim 1, characterized in that the gene codes for a wild type FMDH protein.

3. The DNA fragment according to any one of claims 1 or 2, characterized in that it has been modified by recombinant DNA technologies, while retaining its promoter function.

4. The DNA fragment according to claim 1, characterized in that it comprises the 5' region of the nucleotide sequence shown in Figure 5.

5. The DNA fragment according to any one of claims 1 to 4, characterized in that it further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.

6. The DNA fragment according to claim 5, characterized in that the said foreign gene is selected from genes encoding:
   (a) Hepatitis B Virus S1-S2-S antigen
   (b) Hepatitis B Virus S-antigen
   (c) alpha-amylase from Schwanniomyces castellii
   (d) glucoamylase from Schwanniomyces castellii
   (e) invertase from Saccharomyces cerevisiae.

7. The DNA fragment according to any one of claims 1 to 6, characterized in that it comprises a DNA sequence coding for a secretory signal.

8. The DNA fragment according to claim 7, characterized in that the secretory signal is selected from: Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α-amylase and glucoamylase signals, Saccharomyces cerevisiae α-factor and invertase signals.

9. The DNA fragment according to any one of claims 1 to 8, characterized in that it has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

10. A recombinant vector, characterized in that it contains the DNA fragment according to any one of claims 1 to 9.

11. A microorganism characterized in that it comprises a vector according to claim 10.

12. The microorganism according to claim 11, characterized in that it is a methylotrophic yeast.

13. The microorganism according to claim 12, characterized in that the yeast is selected from the genera Candida, Hansenula or Pichia.

14. The microorganism according to any one of claims 11 to 13, characterized in that it has received the vector according to claim 10 by transformation.

15. The microorganism according to any one of claims 11 to 14, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.

16. The microorganism according to any one of claims 11 to 15, characterized in that it tolerates high concentrations of foreign protein.

17. A process for producing a useful substance, characterized in that a microorganism according to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a manner known in the art.

18. A process for preparing a DNA fragment, wherein said DNA fragment comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment, by isolating said DNA fragment in a manner known per se.

19. The process according to claim 18,characterized in that the gene codes for a wild type FMDH protein.

20. The process according to any one of claims 18 or 19, characterized in that the DNA fragment has been modified by recombinant DNA technologies, while retaining its promoter function.

21. The process according to claim 18,characterized in that the DNA fragment comprises the 5' region of the nucleotide sequence shown in Figure 5.

22. The process according to any one of claims 18 to 21, characterized in that the DNA fragment further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.

23. The process according to claim 22, characterized in that the said foreign gene is selected from genes encoding:
    (a) Hepatitis B Virus S1-S2-S antigen
    (b) Hepatitis B Virus S-antigen
    (c) alpha-amylase from Schwanniomyces castellii
    (d) glucoamylase from Schwanniomyces castellii
    (e) invertase from Saccharomyces cerevisiae.

24. The process according to any one of claims 18 to 23, characterized in that the DNA fragment comprises a DNA sequence coding for a secretory signal.

25. The process according to claim 24, characterized in that the secretory signal is selected from:
Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α-amylase and glucoamylase signals, Saccharomyces cerevisiae α-factor and invertase signals.

26. The process according to any one of claims 18 to 25, characterized in that the DNA fragment has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

27. A process for preparing a recombinant vector, characterized in that the DNA fragment produced according to any one of claims 18 to 26 is inserted into a cloning vector.

28. A process for preparing microorganism characterized in that a vector according to claim 27 is introduced into said microorganism.

29. The process according to claim 28, characterized in that the microorganism is a methylotrophic yeast.

30. The process according to claim 29, characterized in that the yeast is selected from the genera Candida, Hansenula or Pichia.

31. The process according to any one of claims 28 to 30, characterized in that the vector produced according to claim 27 has been introduced by transformation.

32. The process according to any one of claims 28 to 31, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.

33. The process according to any one of claims 28 to 32, characterized in that the microorganism tolerates high concentrations of foreign protein.

34. A process for producing a useful substance, characterized in that a microorganism produced according to any one of claims 18 to 33 is cultured and the useful substance is recovered and purified in a manner known in the art.

Claims for the following Contracting State : ES

1. A process for preparing a DNA fragment, wherein said DNA fragment comprises a promoter region of a gene coding for a protein derived from a methylotrophic yeast and having formate dehydrogenase (FMDH) activity, said gene being identical or equivalent to a FMDH gene being obtainable from the Hansenula polymorpha genome, wherein the FMDH gene is located on a 3.5 kb BamHI-HindIII fragment, by isolating said DNA fragment in a manner known per se.

2. The process according to claim 1, characterized in that the gene codes for a wild type FMDH protein.

3. The process according to any one of claims 1 or 2, characterized in that the DNA fragment has been modified by recombinant DNA technologies, while retaining its promoter function.

4. The process according to claim 1, characterized in that the DNA fragment comprises the 5' region of the nucleotide sequence shown in Figure 5.

5. The process according to any one of claims 1 to 4, characterized in that the DNA fragment further comprises at least one DNA sequence encoding a foreign gene under the transcriptional control of the promoter region.

6. The process according to claim 5, characterized in that the said foreign gene is selected from genes encoding:
   (a) Hepatitis B Virus S1-S2-S antigen
   (b) Hepatitis B Virus S-antigen
   (c) alpha-amylase from Schwanniomyces castellii
   (d) glucoamylase from Schwanniomyces castellii
   (e) invertase from Saccharomyces cerevisiae.

7. The process according to any one of claims 1 to 6, characterized in that the DNA fragment comprises a DNA sequence coding for a secretory signal.

8. The process according to claim 7, characterized in that the secretory signal is selected from: Hansenula polymorpha membrane translocation signals, preferably those from peroxisomal proteins methanol oxidase and dihydroxyacetone synthase, Schwanniomyces castellii α-amylase and glucoamylase signals, Saccharomyces cerevisiae α-factor and invertase signals.

9. The process according to any one of claims 1 to 8, characterized in that the DNA fragment has been obtained from natural DNA and/or cDNA and/or chemically synthesized DNA, wherein the promoter region has been obtained from natural DNA and/or chemically synthesized DNA.

10. A process for preparing a recombinant vector, characterized in that the DNA fragment produced according to any one of claims 1 to 9 is inserted into a cloning vector.

11. A process for preparing microorganism characterized in that a vector according to claim 10 is introduced into said microorganism.

12. The process according to claim 11, characterized in that the microorganism is a methylotrophic yeast.

13. The process according to claim 12, characterized in that the yeast is selected from the genera Candida, Hansenula or Pichia.

14. The process according to any one of claims 11 to 13, characterized in that the vector produced according to claim 10 has been introduced by transformation.

15. The process according to any one of claims 11 to 14, characterized in that the vector is integrated into the genome of the microorganism or maintained as an extrachromosomal DNA molecule.

16. The process according to any one of claims 11 to 15, characterized in that the microorganism tolerates high concentrations of foreign protein.

17. A process for producing a useful substance, characterized in that a microorganism produced according to any one of claims 11 to 16 is cultured and the useful substance is recovered and purified in a manner known in the art.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. DNA-Fragment, dadurch gekennzeichnet, daß es einen Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist.

2. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

3. DNA-Fragment nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß es durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.

4. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß es den 5'-Bereich der in Figur 5 gezeigten Nukleotidsequenz umfaßt.

5. DNA-Fragment nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß es weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.

6. DNA-Fragment nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen ausgewählt ist aus Genen, die kodieren für:
   (a) Hepatitis B Virus S1-S2-S Antigen

(b) Hepatitis B Virus S-Antigen
(c) alpha-Amylase aus Schwanniomyces castellii
(d) Glucoamylase aus Schwanniomyces castellii
(e) Invertase aus Saccharomyces cerevisiae.

7. DNA-Fragment nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß es eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.

8. DNA-Fragment nach Anspruch 7, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus:
Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroacetonsynthase, Schwanniomyces castellii α-Amylase- und Glucoamylasesignalen, Saccharomyces cerevisiae α-Faktor- und Invertasesignalen.

9. DNA-Fragment nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß es aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten ist.

10. Rekombinanter Vektor, dadurch gekennzeichnet, daß er das DNA-Fragment nach einem der Ansprüche 1 bis 9 enthält.

11. Mikroorganismus, dadurch gekennzeichnet, daß er einen Vektor nach Anspruch 10 umfaßt.

12. Mikroorganismus nach Anspruch 11, dadurch gekennzeichnet, daß er eine methylotrophe Hefe ist.

13. Mikroorganismus nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.

14. Mikroorganismus nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß er den Vektor nach Anspruch 10 durch Transformation erhalten hat.

15. Mikroorganismus nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert ist oder als extrachromosomales DNA-Molekül erhalten bleibt.

16. Mikroorganismus nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß er hohe Konzentrationen von fremdem Protein toleriert.

17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein Mikroorganismus nach einem der Ansprüche 11 bis 16 kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

Patentansprüche für folgenden Vertragsstaat : AT

1. DNA-Fragment, dadurch gekennzeichnet, daß es einen Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist.

2. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

3. DNA-Fragment nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß es durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.

4. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß es den 5'-Bereich der in Figur 5 gezeigten Nukleotidsequenz umfaßt.

5. DNA-Fragment nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß es weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.

6. DNA-Fragment nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen ausgewählt ist aus Genen, die kodieren für:
   (a) Hepatitis B Virus S1-S2-S Antigen
   (b) Hepatitis B Virus S-Antigen
   (c) alpha-Amylase aus Schwanniomyces castellii
   (d) Glucoamylase aus Schwanniomyces castellii
   (e) Invertase aus Saccharomyces cerevisiae.

7. DNA-Fragment nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß es eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.

8. DNA-Fragment nach Anspruch 7, dadurch gekennzeichnet, daß das Sektretionssignal ausgewählt ist aus:
   Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroacetonsynthase, Schwanniomyces castellii α-Amylase- und Glucoamylasesignalen, Saccharomyces cerevisiae α-Faktor- und Invertasesignalen.

9. DNA-Fragment nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß es aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten ist.

10. Rekombinanter Vektor, dadurch gekennzeichnet, daß er das DNA-Fragment nach einem der Ansprüche 1 bis 9 enthält.

11. Mikroorganismus, dadurch gekennzeichnet, daß er einen Vektor nach Anspruch 10 umfaßt.

12. Mikroorganismus nach Anspruch 11, dadurch gekennzeichnet, daß er eine methylotrophe Hefe ist.

13. Mikroorganismus nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.

14. Mikroorganismus nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß er den Vektor nach Anspruch 10 durch Transformation erhalten hat.

15. Mikroorganismus nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert ist oder als extrachromosomales DNA-Molekül erhalten bleibt.

16. Mikroorganismus nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß er hohe Konzentrationen von fremdem Protein toleriert.

17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein Mikroorganismus nach einem der Ansprüche 11 bis 16 kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

18. Verfahren zum Herstellen eines DNA-Fragmentes, wobei das DNA-Fragment den Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist, indem das DNA-Fragment auf übliche Weise isoliert wird.

19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

20. Verfahren nach einem der Ansprüche 18 oder 19, dadurch ge- kennzeichnet, daß das DNA-Fragment durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.

21. Verfahren nach Anspruch 18, dadurch gekennzeichnet, daß das DNA-Fragment den 5'-Bereich der in Figur 5 gezeigten Nukleotidssequenz umfaßt.

22. Verfahren nach einem der Ansprüche 18 bis 21, dadurch gekennzeichnet, daß das DNA-Fragment weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.

23. Verfahren nach Anspruch 22, dadurch gekennzeichnet, daß das fremde Gen aus den Genen ausgewählt ist, die kodieren für:
(a) Hepatitis B Virus S1-S2-S-Antigen
(b) Hepatitis B Virus S-Antigen
(c) alpha-Amylase aus Schwanniomyces castellii
(d) Glucoamylase aus Schwanniomyces castellii
(e) Invertase aus Saccharomyces cerevisiae.

24. Verfahren nach einem der Ansprüche 18 bis 23, dadurch gekennzeichnet, daß das DNA-Fragment eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.

25. Verfahren nach Anspruch 24, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus: Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroxyacetonsynthase, Schwanniomyces castellii α-Amylase- und Glucoamylasessignalen, Saccharomyces cerevisiae α-Faktor- und Invertasesignalen.

26. Verfahren nach einem der Ansprüche 18 bis 25, dadurch gekennzeichnet, daß das DNA-Fragment aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten worden ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten worden ist.

27. Verfahren zum Herstellen eines rekombinanten Vektors, dadurch gekennzeichnet, daß das nach einem der Ansprüche 18 bis 26 erzeugte DNA-Fragment in einen Klonierungsvektor insertiert wird.

28. Verfahren zum Herstellen eines Mikroorganismus, dadurch gekennzeichnet, daß ein Vektor nach Anspruch 27 in den Mikroorganismus eingeführt wird.

29. Verfahren nach Anspruch 28, dadurch gekennzeichnet, daß der Mikroorganismus eine methylotrophe Hefe ist.

30. Verfahren nach Anspruch 29, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.

31. Verfahren nach einem der Ansprüche 28 bis 30, dadurch gekennzeichnet, daß der nach Anspruch 27 erzeugte Vektor durch Transformation eingeführt wird.

32. Verfahren nach einem der Ansprüche 28 bis 31, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert wird oder als ein extrachromosomales DNA-Molekül erhalten bleibt.

33. Verfahren nach einem der Ansprüche 28 bis 32, dadurch gekennzeichnet, daß der Mikroorganismus hohe Konzentrationen von fremdem Protein toleriert.

34. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein nach einem der Ansprüche 18 bis 33 erzeugter Mikroorganismus kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zum Herstellen eines DNA-Fragmentes, wobei das DNA-Fragment den Promoterbereich eines für ein Protein mit Formiatdehydrogenase (FMDH) Aktivität kodierenden Genes umfaßt, das von einer methylotrophen Hefe abgeleitet ist, wobei das Gen einem FMDH-Gen identisch oder äquivalent ist, das aus dem Hansenula polymorpha Genom erhältlich ist, worin das FMDH-Gen auf einem 3,5 kb BamHI-HindIII-Fragment angeordnet ist, indem das DNA-Fragment auf übliche Weise isoliert wird.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Gen für ein Wildtyp-FMDH-Protein kodiert.

3. Verfahren nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß das DNA-Fragment durch rekombinante DNA-Technologie modifiziert worden ist, während es seine Promoterfunktion behält.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das DNA-Fragment den 5'-Bereich der in Figur 5 gezeigten Nukleotidssequenz umfaßt.

5. Verfahren nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß das DNA-Fragment weiter mindestens eine für ein fremdes Gen kodierende DNA-Sequenz unter der Transkriptionskontrolle des Promoterbereiches umfaßt.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß das fremde Gen aus den Genen ausgewählt ist, die kodieren für:
   (a) Hepatitis B Virus S1-S2-S-Antigen
   (b) Hepatitis B Virus S-Antigen
   (c) alpha-Amylase aus Schwanniomyces castellii
   (d) Glucoamylase aus Schwanniomyces castellii
   (e) Invertase aus Saccharomyces cerevisiae.

7. Verfahren nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß das DNA-Fragment eine für ein Sekretionssignal kodierende DNA-Sequenz umfaßt.

8. Verfahren nach Anspruch 7, dadurch gekennzeichnet, daß das Sekretionssignal ausgewählt ist aus: Hansenula polymorpha Membran-Translokationssignalen, bevorzugt solchen von den peroxisomalen Proteinen Methanoloxidase und Dihydroxyacetonsynthase, Schwanniomyces castellii α-Amylase- und Glucoamylasesignalen, Saccharomyces cerevisiae α-Faktor- und Invertasesignalen.

9. Verfahren nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß das DNA-Fragment aus natürlicher DNA und/oder cDNA und/oder chemisch synthetisierter DNA erhalten worden ist, wobei der Promoterbereich aus natürlicher DNA und/oder chemisch synthetisierter DNA erhalten worden ist.

10. Verfahren zum Herstellen eines rekombinanten Vektors, dadurch gekennzeichnet, daß das nach einem der Ansprüche 1 bis 9 erzeugte DNA-Fragment in einen Klonierungsvektor insertiert wird.

11. Verfahren zum Herstellen eines Mikroorganismus, dadurch gekennzeichnet, daß ein Vektor nach Anspruch 10 in den Mikroorganismus eingeführt wird.

12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß der Mikroorganismus eine methylotrophe Hefe ist.

13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, daß die Hefe aus den Gattungen Candida, Hansenula oder Pichia ausgewählt ist.

14. Verfahren nach einem der Ansprüche 11 bis 13, dadurch gekennzeichnet, daß der nach Anspruch 10 erzeugte Vektor durch Transformation eingeführt wird.

15. Verfahren nach einem der Ansprüche 11 bis 14, dadurch gekennzeichnet, daß der Vektor in das Genom des Mikroorganismus integriert wird oder als ein extrachromosomales DNA-Molekül erhalten

bleibt.

16. Verfahren nach einem der Ansprüche 11 bis 15, dadurch gekennzeichnet, daß der Mikroorganismus hohe Konzentrationen von fremdem Protein toleriert.

17. Verfahren zum Herstellen einer nützlichen Substanz, dadurch gekennzeichnet, daß ein nach einem der Ansprüche 11 bis 16 erzeugter Mikroorganismus kultiviert wird und die nützliche Substanz auf übliche Weise gewonnen und gereinigt wird.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Fragment d'ADN caractérisé en ce qu'il comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment *Bam*HI-*Hind*III de 3,5 kb.

2. Fragment d'ADN selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

3. Fragment d'ADN selon l'une quelconque des revendications 1 ou 2, caractérisé en ce qu'il est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

4. Fragment d'ADN selon la revendication 1, caractérisé en ce qu'il comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

5. Fragment d'ADN selon l'une quelconque des revendications 1 à 4, caractérisé en ce qu'il comprend en outre au moins une séquence d'ADN codant un gène étranger sous le contrôle de transcription de la région promotrice.

6. Fragment d'ADN selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant :
   (a) l'antigène S1-S2-S du virus de l'hépatite B
   (b) l'antigène S du virus de l'hépatite B
   (c) une alpha-amylase de *Schwanniomyces castellii*
   (d) une glucoamylase de *Schwanniomyces castellii*
   (e) une invertase de *Saccharomyces cerevisiae*.

7. Fragment d'ADN selon l'une quelconque des revendications 1 à 6, caractérisé en ce qu'il comprend une séquence d'ADN codant pour un signal sécrétoire.

8. Fragment d'ADN selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des α-amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α-facteur et invertase de *Saccharomyces cerevisiae*.

9. Fragment d'ADN selon l'une quelconque des revendications 1 à 8, caractérisé en ce qu'il est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

10. Vecteur recombinant, caractérisé en ce qu'il contient le fragment d'ADN selon l'une quelconque des revendications 1 à 9.

11. Micro-organisme caractérisé en ce qu'il comprend un vecteur selon la revendication 10.

12. Micro-organisme selon la revendication 11, caractérisé en ce que c'est une levure méthylotrophe.

13. Micro-organisme selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.

14. Micro-organisme selon l'une quelconque des revendications 11 à 13, caractérisé en ce qu'il reçoit le vecteur selon la revendication 10 par transformation.

15. Micro-organisme selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du microorganisme ou maintenu comme molécule d'ADN extrachromosomique.

16. Micro-organisme selon l'une quelconque des revendications 11 à 15, caractérisé en ce qu'il tolère des concentrations élevées de protéine étrangère.

17. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

Revendications pour l'Etat contractant suivant : AT

1. Fragment d'ADN caractérisé en ce qu'il comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb.

2. Fragment d'ADN selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

3. Fragment d'ADN selon l'une quelconque des revendications 1 ou 2, caractérisé en ce qu'il est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

4. Fragment d'ADN selon la revendication 1, caractérisé en ce qu'il comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

5. Fragment d'ADN selon l'une quelconque des revendications 1 à 4, caractérisé en ce qu'il comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.

6. Fragment d'ADN selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :
   (a) l'antigène S1-S2-S du virus de l'hépatite B
   (b) l'antigène S du virus de l'hépatite B
   (c) l'alpha-amylase de *Schwanniomyces castellii*
   (d) la glucoamylase de *Schwanniomyces castellii*
   (e) l'invertase de *Saccharomyces cerevisiae.*

7. Fragment d'ADN selon l'une quelconque des revendications 1 à 6, caractérisé en ce qu'il comprend une séquence d'ADN codant pour un signal sécrétoire.

8. Fragment d'ADN selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des α-amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α-facteur et invertase de *Saccharomyces cerevisiae*.

9. Fragment d'ADN selon l'une quelconque des revendications 1 à 8, caractérisé en ce qu'il est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

10. Vecteur recombinant, caractérisé en ce qu'il contient le fragment d'ADN selon l'une quelconque des revendications 1 à 9.

11. Micro-organisme caractérisé en ce qu'il comprend un vecteur selon la revendication 10.

12. Microorganisme selon la revendication 11, caractérisé en ce que c'est une levure méthylotrophe.

13. Micro-organisme selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.

14. Micro-organisme selon l'une quelconque des revendications 11 à 13, caractérisé en ce qu'il reçoit le vecteur selon la revendication 10 par transformation.

15. Micro-organisme selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du microorganisme ou maintenu comme molécule d'ADN extrachromosomique.

16. Micro-organisme selon l'une quelconque des revendications 11 à 15, caractérisé en ce qu'il tolère des concentrations élevées de protéine étrangère.

17. Procédé pour préparer une substance utile, caractérisé en ce qu un micro-organisme selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

18. Procédé pour préparer un fragment d'ADN, dans lequel ledit fragment d'ADN comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gène de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb, en isolant ledit fragment d'ADN d'une manière connue en soi.

19. Procédé selon la revendication 18, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

20. Procédé selon l'une quelconque des revendications 18 ou 19, caractérisé en ce que le fragment d'ADN est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

21. Procédé selon la revendication 18, caractérisé en ce que le fragment d'ADN comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

22. Procédé selon l'une quelconque des revendications 18 à 21, caractérisé en ce que le fragment d'ADN comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.

23. Procédé selon la revendication 22, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :
    (a) l'antigène S1-S2-S du virus de l'hépatite B
    (b) l'antigène S du virus de l'hépatite B
    (c) l'alpha-amylase de *Schwanniomyces castellii*
    (d) la glucoamylase de *Schwanniomyces castellii*
    (e) l'invertase de *Saccharomyces cerevisiae*.

24. Procédé selon l'une quelconque des revendications 18 à 23, caractérisé en ce que le fragment d'ADN comprend une séquence d'ADN codant pour un signal sécrétoire.

25. Procédé selon la revendication 24, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des

α-amylase et glucoamylase de *Schwanniomyces castellii*, les signaux des α-facteur et invertase de *Saccharomyces cerevisiae*.

26. Procédé selon l'une quelconque des revendications 18 à 25, caractérisé en ce que le fragment d'ADN est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

27. Procédé pour préparer un vecteur recombinant, caractérisé en ce que le fragment d'ADN obtenu selon l'une quelconque des revendications 18 à 26 est inséré dans un vecteur clonant.

28. Procédé pour préparer un micro-organisme caractérisé en ce qu'un vecteur selon la revendication 27 est introduit dans ledit micro-organisme.

29. Procédé selon la revendication 28, caractérisé en ce que le micro-organisme est une levure méthylotrophe.

30. Procédé selon la revendication 29, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia*.

31. Procédé selon l'une quelconque des revendications 28 à 30, caractérisé en ce que le vecteur obtenu selon la revendication 27 est introduit par transformation.

32. Procédé selon l'une quelconque des revendications 28 à 31, caractérisé en ce que le vecteur est intégré dans le génome du micro-organisme ou maintenu comme molécule d'ADN extrachromosomique.

33. Procédé selon l'une quelconque des revendications 28 à 32, caractérisé en ce que le micro-organisme tolère des concentrations élevées de protéine étrangère.

34. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme obtenu selon l'une quelconque des revendications 18 à 33 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour préparer un fragment d'ADN, dans lequel ledit fragment d'ADN comprend une région promotrice d'un gène codant pour une protéine dérivée d'une levure méthylotrophe et ayant l'activité de la formate déshydrogénase (FMDH), ledit gène étant identique ou équivalent à un gène de FMDH, pouvant être obtenu à partir du génome de *Hansenula polymorpha*, dans lequel le gêne de FMDH est situé sur un fragment BamHI-HindIII de 3,5 kb, en isolant ledit fragment d'ADN d'une manière connue en soi.

2. Procédé selon la revendication 1, caractérisé en ce que le gène code pour une protéine de FMDH de type sauvage.

3. Procédé selon l'une quelconque des revendications 1 ou 2, caractérisé en ce que le fragment d'ADN est modifié par les techniques d'ADN recombinant, tout en conservant sa fonction de promoteur.

4. Procédé selon la revendication 1, caractérisé en ce que le fragment d'ADN comprend la région 5' de la séquence de nucléotides présentée à la figure 5.

5. Procédé selon l'une quelconque des revendications 1 à 4, caractérisé en ce que le fragment d'ADN comprend en outre au moins une séquence d'ADN codant pour un gène étranger sous le contrôle de transcription de la région promotrice.

6. Procédé selon la revendication 5, caractérisé en ce que ledit gène étranger est choisi parmi les gènes codant pour :
   (a) l'antigène S1-S2-S du virus de l'hépatite B

(b) l'antigène S du virus de l'hépatite B
(c) l'alpha-amylase de *Schwanniomyces castellii*
(d) la glucoamylase de *Schwanniomyces castellii*
(e) l'invertase de *Saccharomyces cerevisiae.*

7. Procédé selon l'une quelconque des revendications 1 à 6, caractérisé en ce que le fragment d'ADN comprend une séquence d'ADN codant pour un signal sécrétoire.

8. Procédé selon la revendication 7, caractérisé en ce que le signal sécrétoire est choisi parmi : les signaux de translocation de la membrane de *Hansenula polymorpha*, de préférence ceux des protéines peroxisomales, de la méthanol oxydase et de la dihydroxyacétone synthase, les signaux des α-amylase et glucoamylase de *Schwanniomyces castellii,* les signaux des α-facteur et invertase de *Saccharomyces cerevisiae.*

9. Procédé selon l'une quelconque des revendications 1 à 8, caractérisé en ce que le fragment d'ADN est obtenu à partir d'ADN naturel et/ou d'ADNc et/ou d'ADN synthétisé par voie chimique, dans lequel la région promotrice est obtenue à partir d'ADN naturel et/ou d'ADN synthétisé par voie chimique.

10. Procédé pour préparer un vecteur recombinant, caractérisé en ce que le fragment d'ADN obtenu selon l'une quelconque des revendications 1 à 9 est inséré dans un vecteur clonant.

11. Procédé pour préparer un micro-organisme caractérisé en ce qu'un vecteur selon la revendication 10 est introduit dans ledit micro-organisme.

12. Procédé selon la revendication 11, caractérisé en ce que le micro-organisme est une levure méthylotrophe.

13. Procédé selon la revendication 12, caractérisé en ce que la levure est choisie parmi les genres *Candida*, *Hansenula* ou *Pichia.*

14. Procédé selon l'une quelconque des revendications 11 à 13, caractérisé en ce que le vecteur obtenu selon la revendication 10 est introduit par transformation.

15. Procédé selon l'une quelconque des revendications 11 à 14, caractérisé en ce que le vecteur est intégré dans le génome du micro-organisme ou maintenu comme molécule d'ADN extrachromosomique.

16. Procédé selon l'une quelconque des revendications 11 à 15, caractérisé en ce que le micro-organisme tolère des concentrations élevées de protéine étrangère.

17. Procédé pour préparer une substance utile, caractérisé en ce qu'un micro-organisme obtenu selon l'une quelconque des revendications 11 à 16 est cultivé et en ce que la substance utile est récupérée et purifiée d'une manière connue dans l'art.

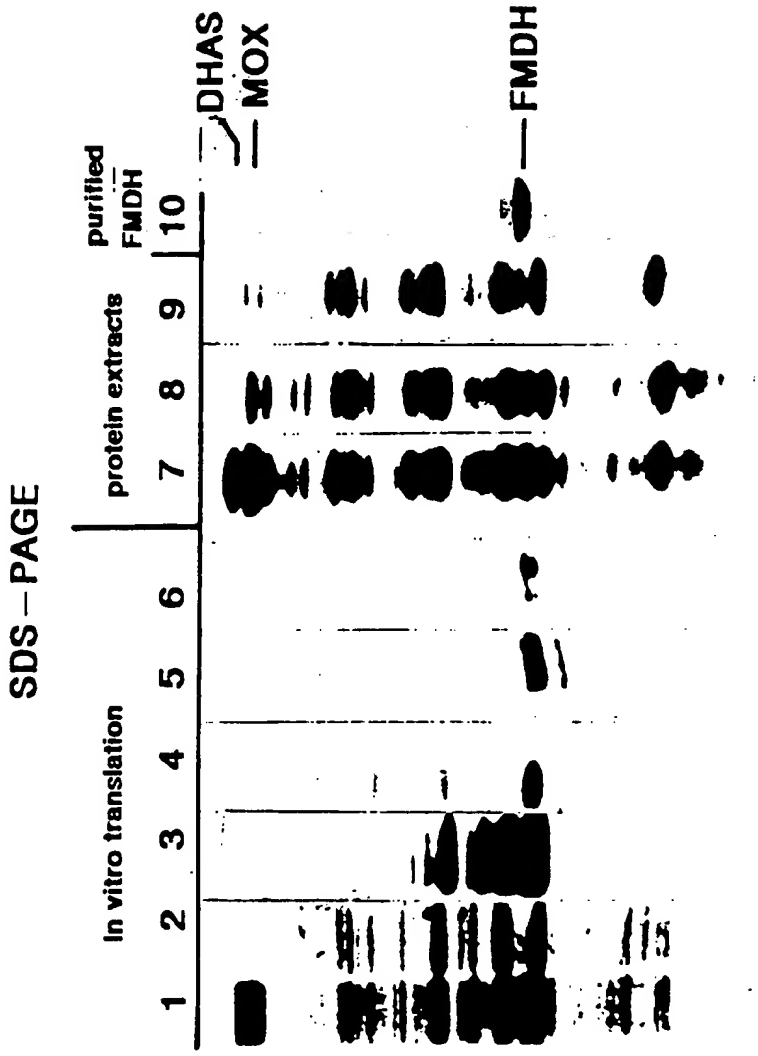


FIG.1.Analysis of protein crude extracts and in vitro translation products by SDS -polyacrylamid gel electrophoresis.

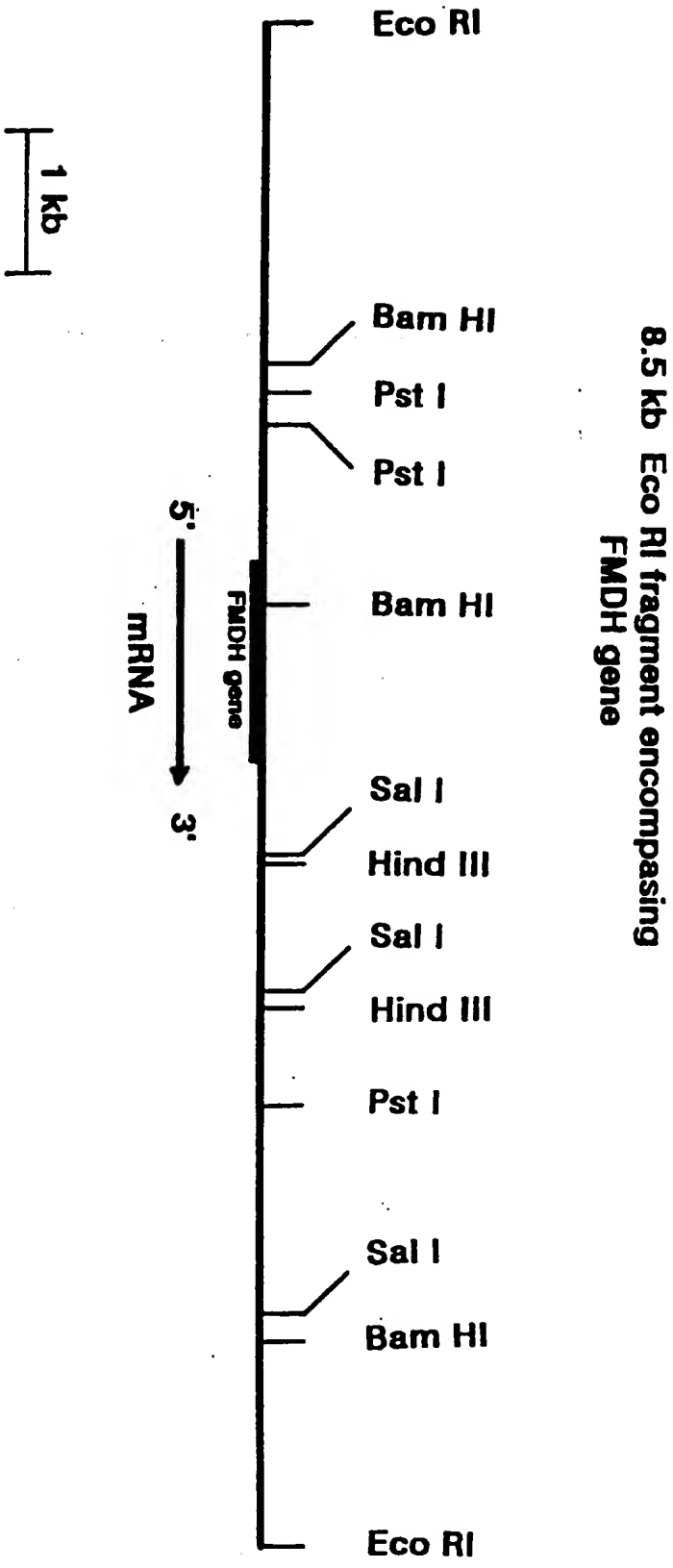


FIG.2.Restriction map of DNA fragment encompassing the FMDH gene.

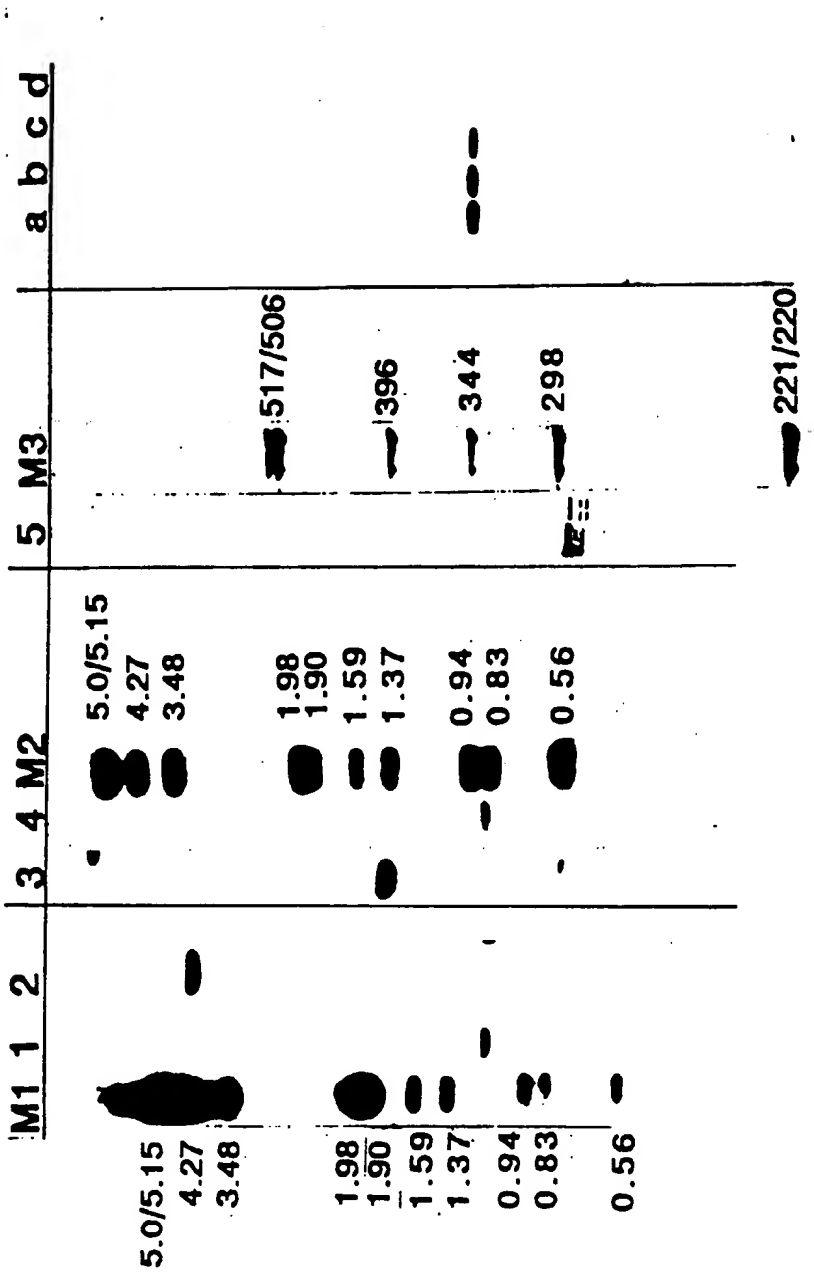


FIG.3 S1-mapping

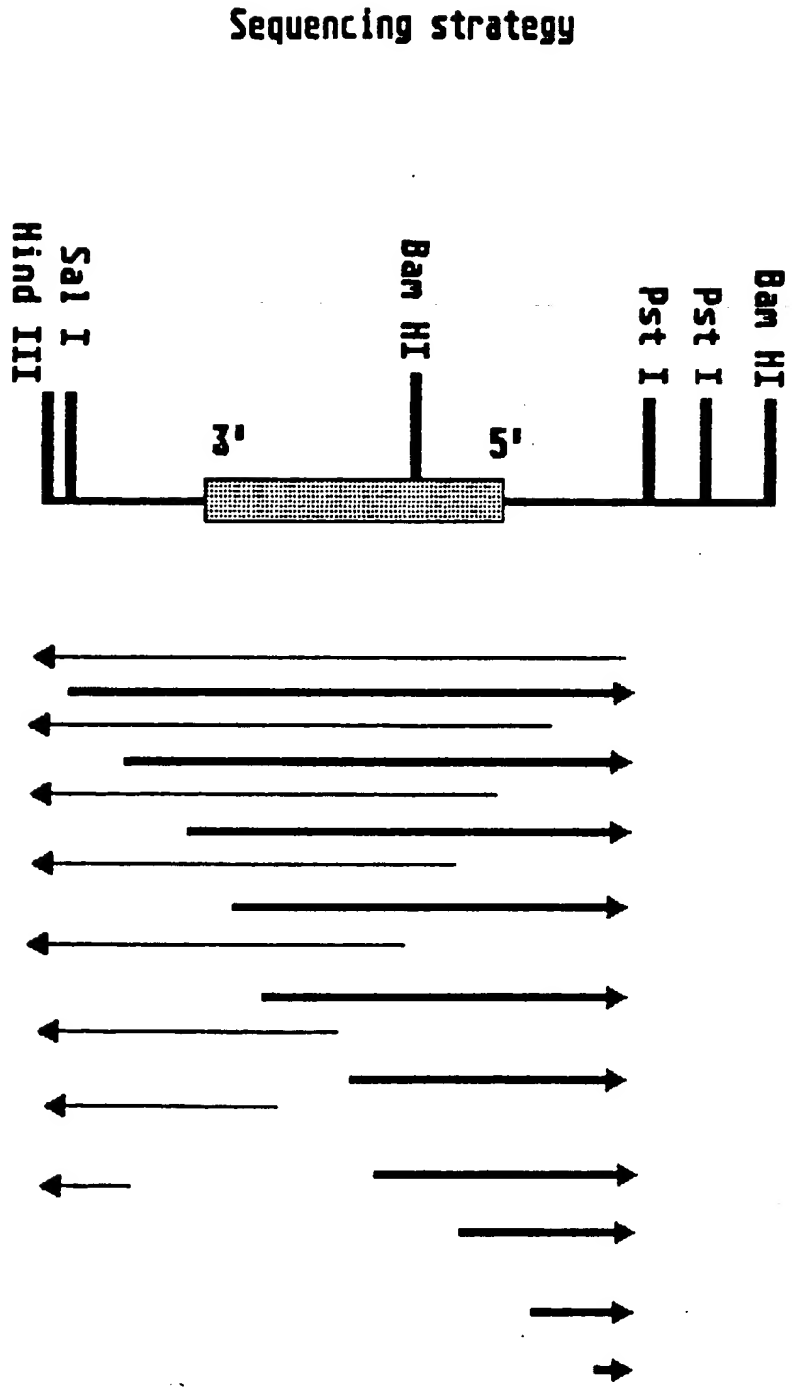


FIG.4 Sequencing strategy - schematic representation

FIG.5a Nucleotide sequence of FMDH gene and its 5',3' control regions.

```
        10         20         30         40         50         60
ATCGCAGAAATGTATCTAAACGCAAACTCCGAGCTGGAAAAATGTTACCGGCGATGCGCG

        70         80         90        100        110        120
GACAATTTAGAGGCGGCAATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCT

       130        140        150        160        170        180
TCGATGGGCCCGAGATCCCACCGCGTTCCTGGGTACCGGGACGTGAGGCAGCGCGACATC

       190        200        210        220        230        240
CTACAAATATACCAGGCGCCAACCGAGTCTCTCGGAAAACACAGCTTCTGGATATCTTCC

       250        260        270        280        290        300
GCGGCGGCGCAACGAGCCAAGAATAGTCCCTGGAGGTGACGGAATATATATGTGTGGAGG

       310        320        330        340        350        360
GTAAATCTGACAGGGTGTAGCAAAGGTAATATTTTCCTAAAACATGCAATCGGCTGCCCC

       370        380        390        400        410        420
GCAACGGGAAAAAGAATGACTTTGGCACTCTTCACCAGAGTGGGGTGTCCCGCTCGTGTG

       430        440        450        460        470        480
TGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAGAAAAACAGCAAGTTCCGGGGTG

       490        500        510        520        530        540
TCTCACTGGTGTCCGCCAATAAGAGGACCGGCAGGCACGGAGTCTACATCAAGCTGTCTC

       550        560        570        580        590        600
CGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGCACTATAAATACCGCC

       610        620        630        640        650        660
TCCTTGCGCTCTCTGCCTTCATCAATCAAATCATGAAGGTTGTACTAGTTCTCTACGACG
                                    MetLysValValLeuValLeuTyrAspA

       670        680        690        700        710        720
CAGGAAAACACGCCCAAGACGAGGAAAGACTCTACGGTTGCACTGAAAACGCCCTTGGTA
laGlyLysHisAlaGlnAspGluGluArgLeuTyrGlyCysThrGluAsnAlaLeuGlyI

       730        740        750        760        770        780
TCAGGGACTGGCTCGAGAAGCAGGGCCACGACGTCGTTGTCACCAGTGACAAGGAGGGGC
leArgAspTrpLeuGluLysGlnGlyHisAspValValValThrSerAspLysGluGlyG

       790        800        810        820        830        840
AGAACTCTGTGCTCGAGAAGAACATCTCGGACGCAGATGTCATCATCTCCACTCCTTTCC
lnAsnSerValLeuGluLysAsnIleSerAspAlaAspValIleIleSerThrProPheH

       850        860        870        880        890        900
ACCCAGCATACATCACCAAGGAGAGAATCGACAAGGCCAAGAAGCTCAAGCTACTGGTGG
isProAlaTyrIleThrLysGluArgIleAspLysAlaLysLysLeuLysLeuLeuValV
```

```
      910        920        930        940        950        960
TTGCCGGAGTGGGATCCGACCACATCGACCTTGACTACATCAACCAGTCCGGCAGAGACA
alAlaGlyValGlySerAspHisIleAspLeuAspTyrIleAsnGlnSerGlyArgAspI

      970        980        990       1000       1010       1020
TTTCTGTGCTGGAGGTGACCGGTTCGAACGTCGTTTCGGTTGCCGAGCACGTTGTGATGA
leSerValLeuGluValThrGlySerAsnValValSerValAlaGluHisValValMetT

     1030       1040       1050       1060       1070       1080
CGATGCTGGTGCTGGTGAGGAACTTTGTTCCTGCTCACGAGCAGATCATCTCTGGCGGCT
hrMetLeuValLeuValArgAsnPheValProAlaHisGluGlnIleIleSerGlyGlyT

     1090       1100       1110       1120       1130       1140
GGAACGTGGCCGAGATCGCCAAGGACTCCTTCGACATCGAGGGCAAGGTCATTGCCACCA
rpAsnValAlaGluIleAlaLysAspSerPheAspIleGluGlyLysValIleAlaThrI

     1150       1160       1170       1180       1190       1200
TCGGAGCAGGCAGAATCGGCTACCGTGTGCTGGAGAGACTTGTGGCCTTCAACCCTAAGG
leGlyAlaGlyArgIleGlyTyrArgValLeuGluArgLeuValAlaPheAsnProLysG

     1210       1220       1230       1240       1250       1260
AGCTGCTCTACTACGACTACCAGTCGCTGTCGAAAGAGGCGGAGGAGAAAGTCGGCGCCC
luLeuLeuTyrTyrAspTyrGlnSerLeuSerLysGluAlaGluGluLysValGlyAlaA

     1270       1280       1290       1300       1310       1320
GCAGAGTCCACGACATCAAGGAGCTGGTTGCCCAGGCCGACATTGTCACGATCAACTGTC
rgArgValHisAspIleLysGluLeuValAlaGlnAlaAspIleValThrIleAsnCysP

     1330       1340       1350       1360       1370       1380
CACTGCACGCCGGCTCGAAGGGCCTGGTGAACGCAGAGCTGCTCAAGCACTTCAAGAAGG
roLeuHisAlaGlySerLysGlyLeuValAsnAlaGluLeuLeuLysHisPheLysLysG

     1390       1400       1410       1420       1430       1440
GCGCCTGGCTCGTCAACACCGCCAGAGGTGCCATCTGCGTGGCCGAGGACGTTGCAGCCG
lyAlaTrpLeuValAsnThrAlaArgGlyAlaIleCysValAlaGluAspValAlaAlaA

     1450       1460       1470       1480       1490       1500
CCGTCAAGAGCGGACAGCTTAGAGGATACGGTGGAGACGTGTGGTTCCCACAGCCAGCTC
laValLysSerGlyGlnLeuArgGlyTyrGlyGlyAspValTrpPheProGlnProAlaP

     1510       1520       1530       1540       1550       1560
CAAAGGACCACCCATGGAGATCCATGGCCAACAAGTACGGTGCTGGCAATGCCATGACTC
roLysAspHisProTrpArgSerMetAlaAsnLysTyrGlyAlaGlyAsnAlaMetThrP

     1570       1580       1590       1600       1610       1620
CGCACTACTCGGGCTCTGTCATTGACGCCCAGGTCAGATACGCGCAGGGCACCAAGAACA
roHisTyrSerGlySerValIleAspAlaGlnValArgTyrAlaGlnGlyThrLysAsnI

     1630       1640       1650       1660       1670       1680
TCCTGGAGTCGTTCTTCACTCAGAAGTTCGACTACAGGCCCCAGGACATCATTCTGCTGA
leLeuGluSerPhePheThrGlnLysPheAspTyrArgProGlnAspIleIleLeuLeuA

     1690       1700       1710       1720       1730       1740
ACGGCAAGTACAAGACCAAGTCGTACGGTGCCGACAAATGAGCGGTCTTGGAGGAGCTGA
snGlyLysTyrLysThrLysSerTyrGlyAlaAspLysEnd

     1750       1760       1770       1780       1790       1800
TTGGATCTAGATGAAATAGGAAATATAATTATGGCTCTACTGCGCTGCGTAAACGTCACT
```

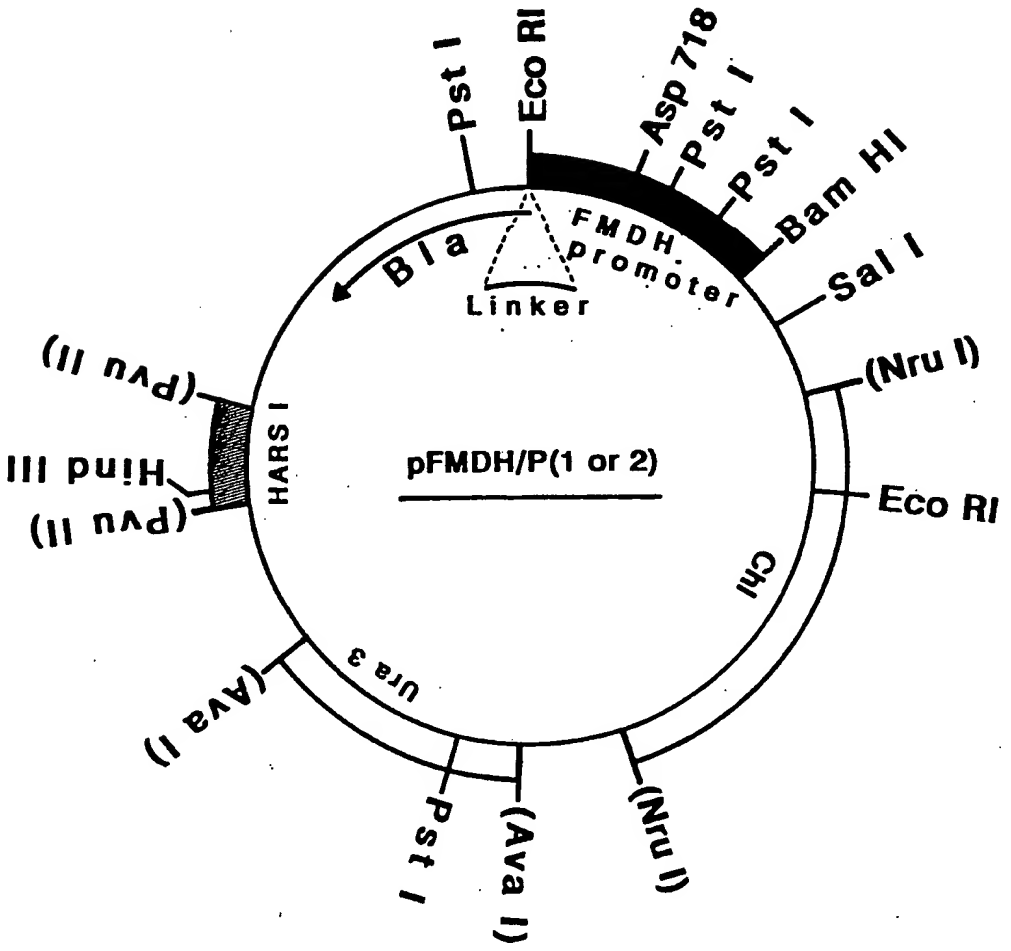
FIG.5δ Nucleotide sequence of FMDH gene and its 5',3' control regions.

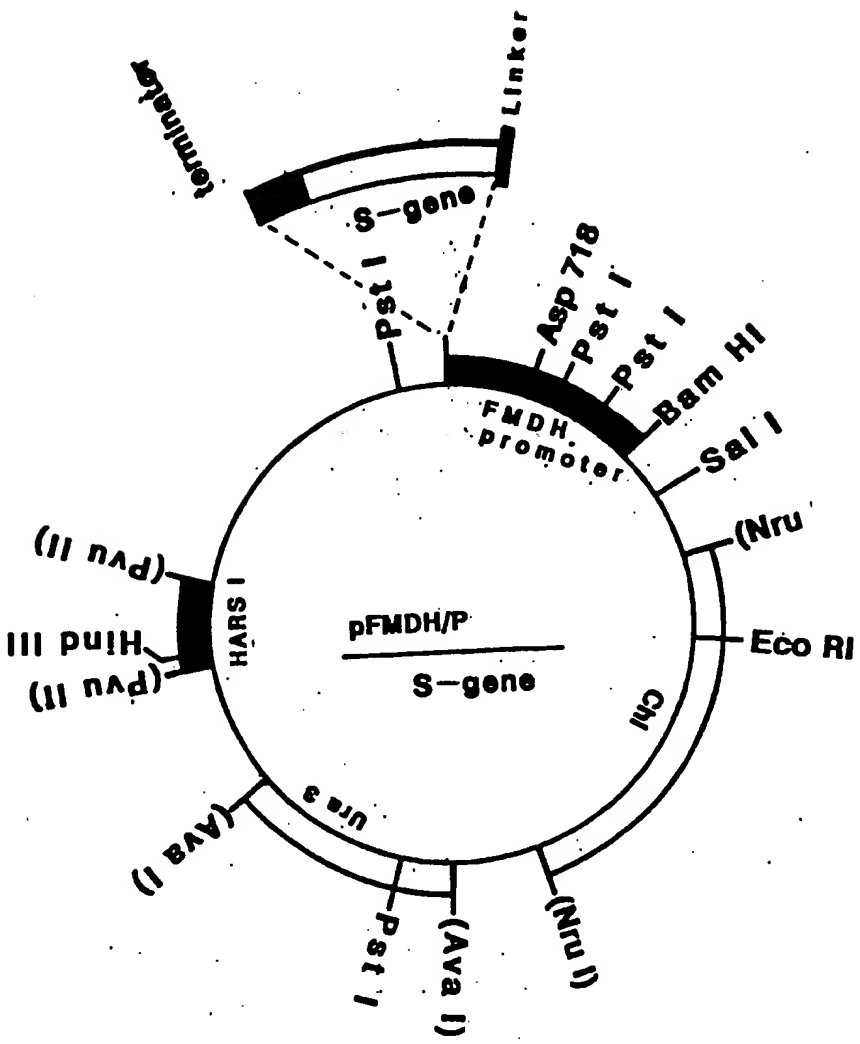
fmdh1

```
      1810       1820       1830       1840       1850       1860
GTAGGCGATTTCGCTTAGCCCAAGTCCGCGATGCGGTCCGACGACACCAGAGCGCGTCCA

      1870       1880       1890       1900       1910       1920
CCTCCTGTGCGCCGCACCGCCCCCAAAGGAGGTTGCGGCTGTGCGGCTCGACGCGACCAA

      1930       1940       1950       1960       1970       1980
AAAAATAAGCGTCAAAAGGAGGTGTCAGGGAAGCACGCCGTGGGGCTCGAGATATATAAA

      1990       2000       2010       2020       2030       2040
GCGCAGCGTAGCTTTGTCTGTCTTGCTAATGAGCGACGACCAAGCCTTGGAAATTTTCCT

      2050       2060       2070       2080       2090       2100
GAAATCCCCCGTCACCCAGGACATGATCCACCACTTGGTGACGGTCACCTTACAGGTTCT

      2110       2120       2130       2140       2150       2160
GCCTTGCGAGTCCTCCAAGACCATCACCCAGAAAGTCAAGTCTTCAGCCGACACAGAGCC

      2170       2180       2190       2200       2210       2220
CGTGCTCAAGACCAAGCCGCTGCCCTCGCTGCATGACTTTCACCAAGCTCGTCCGCTATA

      2230       2240       2250       2260       2270
CCAACGTCTACACGGGAACGTTGATGTCGACCATCGTGTTCCTCAACAGACTXCAA

F
```

FIG.5c Nucleotide sequence of FMDH gene and its 5',3' control regions.

FIG.6 Plasmid containing the fussion of ß-lactamase gene with FMDH promoter.





Linker :

5'-NcoI-BamHI/EcoRI(blunt ended)-3' (in B version)

Fig. 7 Plasmid containing the hepatitis S-gene

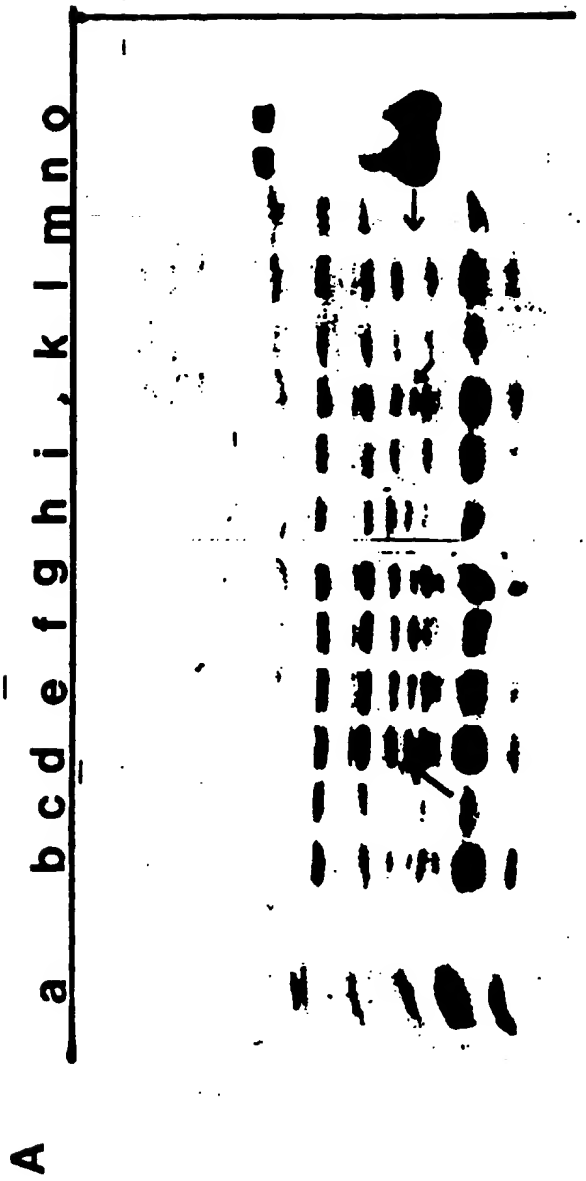


Fig. 8 Western blot-stained by peroxidase/protein A method

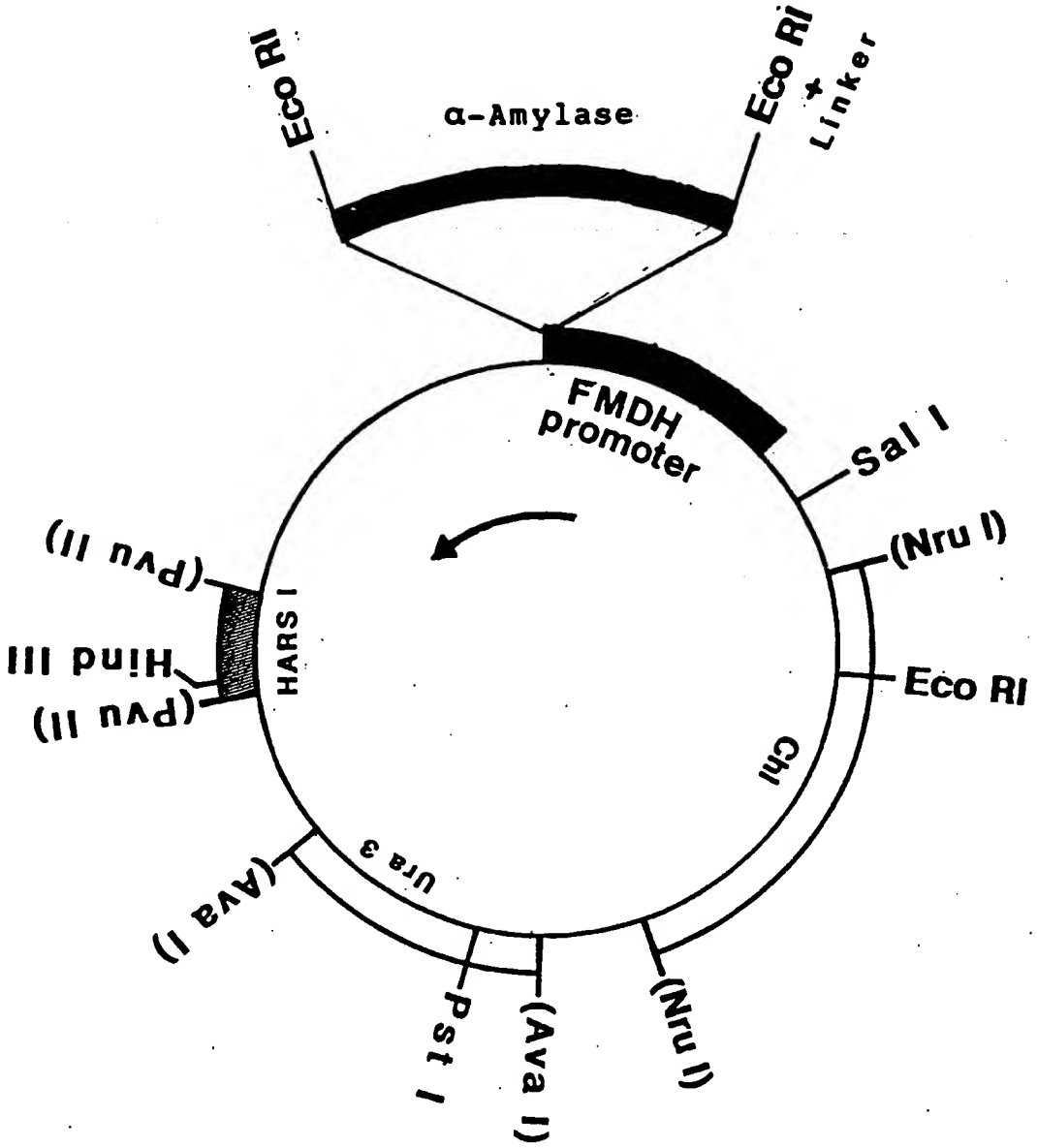


Fig. 9 Plasmid expressing -amylase gene

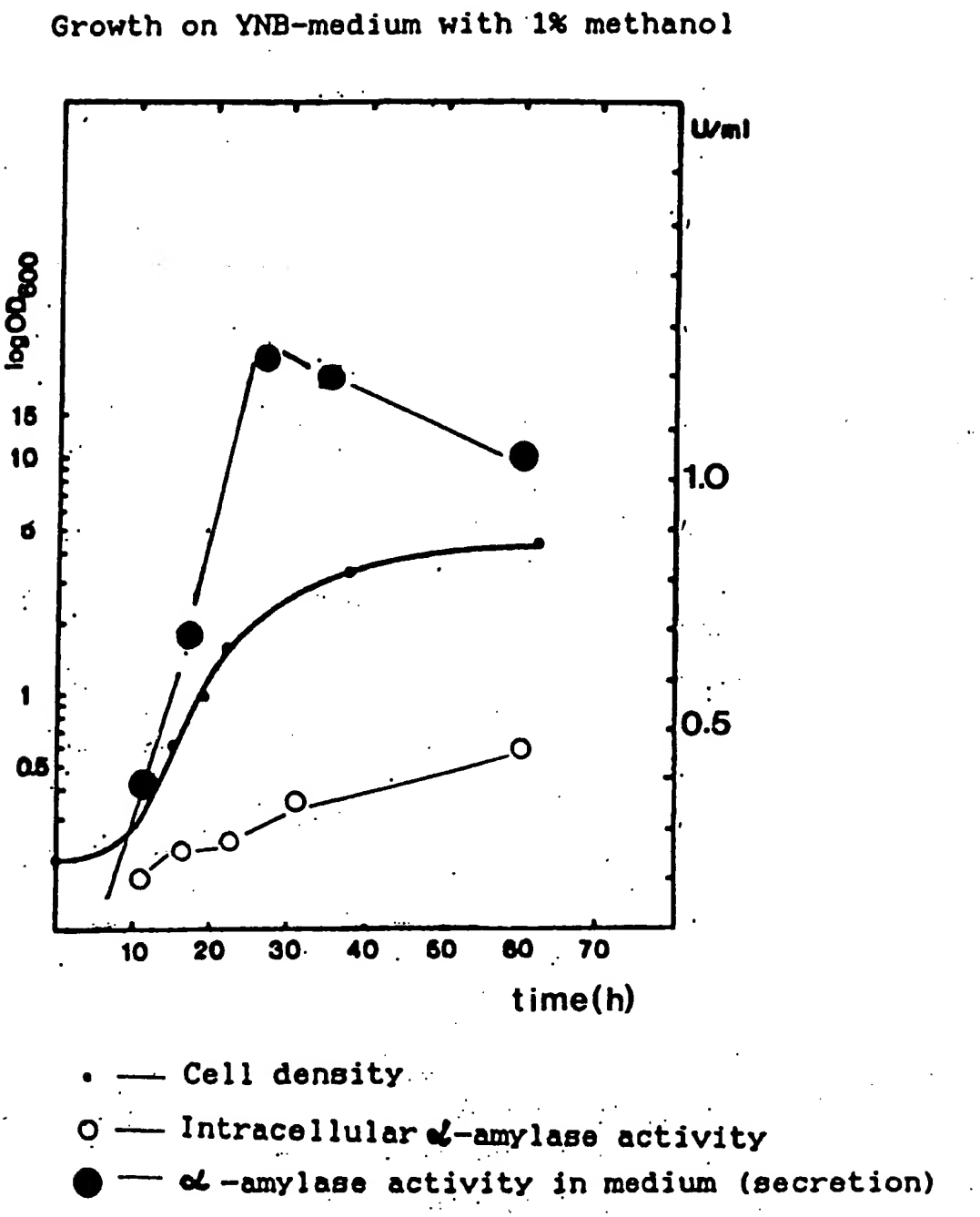


Fig 10 Growth of transformants on medium containing methanol (induction)

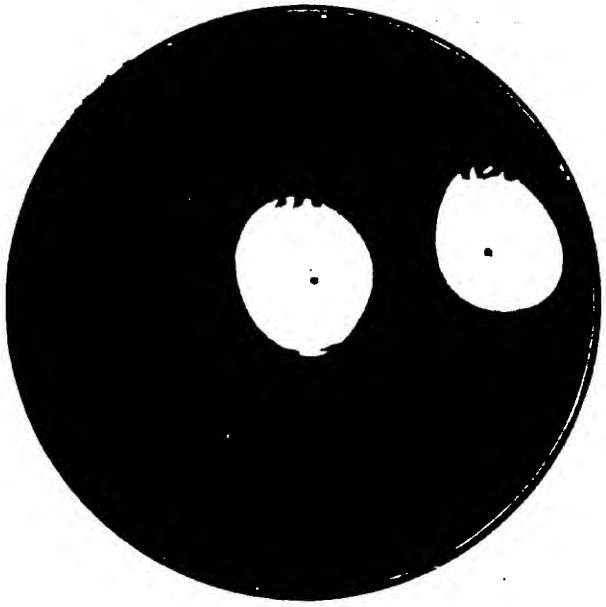


Fig.11.The formation of hallo after applying on the plate 50 ul of the medium from transformants _upper row- and from controluntransformed strain LR9-lower row.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☑ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.